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In re PATENT APPLICATION of
Inventor(s): IMHOF et al.

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Application No.	Country of Origin	Filed
99200746.8	Europe	March 11, 1999

Respectfully submitted,

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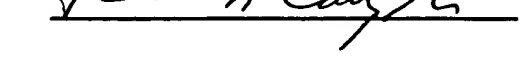
Sig: 

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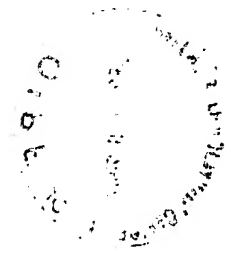

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The function of novel vascular adhesion molecules: A new Ig subfamily

Introduction

Throughout embryonic and early postnatal development, endothelial cells proliferate and differentiate to form new blood vessels via vasculogenesis and angiogenesis (Risau, 1995). In adult organisms the endothelium defines the blood-tissue barrier and consists of non-cycling quiescent cells (Engerman et al. 1967). These polarized cells are linked to each other by tight junctions and adherens junctions to form a continuous layer of cells (Dejana et al.1995). The functions of the endothelial layer consist in the maintenance of tissue homeostasis, fibrinolysis, coagulation, vasotonus, and leukocyte transmigration (Risau, 1995). All these properties are controlled by a fine tuning of the expression and the function of adhesion molecules. Pathological situations such as inflammation, tumor growth, wounding or angiogenesis lead to a temporary change of the number and function of adhesion molecules on the vascular endothelium and this results in altered

homeostasis of the vessel. As an example, tumors increase the local concentration of angiogenic factors which induces a switch from non-cycling quiescent endothelial cells to proliferating endothelium. The angiogenic switch is induced by several factors including IL-8, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), soluble VCAM-1, basic fibroblast growth factor (bFGF), and tumor necrosis factor (TNFα; ref. Koch et al. 1995, Jain et al. 1996). As a result, endothelial cells of existing vessels degrade the extracellular matrix (ECM) and invade the surrounding tissue, which leads to vascularization of tumors (Ekblom et al. 1982, Folkman, 1985). During the angiogenic switch the pattern of endothelial gene expression is modified. For example, treatment of endothelial cells with bFGF or TNFα results in a fourfold increase in $\alpha_v\beta_3$ integrin expression, an adhesion molecule implicated in endothelial cell migration (Brooks et al. 1994). In addition, the angiogenic switch modifies the inflammatory response of endothelium leading to an abnormal migration of leukocytes toward the tumors. Normally, leukocytes extravasate from the blood by adhering to and migrating through endothelium. These mechanisms occur in a multistep process that involves selectins, integrins and Immunoglobulin Superfamily adhesion molecules. In tumor associated endothelium VCAM, ICAM, and selectins have been shown to be down regulated (Griffioen et al. 1996b, Piali et al. 1995). The down regulation of these adhesion molecules may represent a mechanism by which tumors avoid invasion by cytotoxic cells of the immune system (Ning, 1992 and Griffioen et al. 1996a).

Recently, we started to search for new adhesion proteins of the Immunoglobulins Superfamily (Ig Sf), which were transcriptionally regulated in endothelium under the influence of tumors. Our experimental model was the identification transcripts regulated during the coculture of an endothelial cell line with melanoma cells (Piali et al. 1995). To restrict the screening strategy to adhesion molecules of the Ig Sf, we developed a new approach of RNA display termed "Targeted Differential Display" (for ref. see Detlef et al. 1995 and Shima et al. 1995). Partially degenerated primers, designed to target the conserved sequences found in the C_2 domains of Ig Sf members (Williams, 1988) were used to drive the Polymerase Chain Reaction (PCR) based Targeted Differential Display technique. This method allowed the identification of a transcript, down regulated in endothelial cells by confluency in the presence of melanoma or carcinoma cells. The cDNA encoded for a new molecule of the Ig Sf with unusual structural features, named CRAM-1 for Confluency Regulated Adhesion Molecule.

The recent description of a structurally related molecule, JAM, implicated in leukocyte transmigration, suggested the existence of a new family of adhesion molecules in which JAM and CRAM-1 were the prototypes. Sequence comparison with EST databases allowed the cloning of CRAM-2, a third member of this molecular family (**Annex I**). The comparative tissue distribution of the transcripts encoding JAM, CRAM-1 and CRAM-2 showed a preferential expression of these molecules in endothelial and epithelial compartments suggesting a role in the maintenance of cell-cell contacts. These cell-cell

interactions of quiescent endothelial cells regulate the vascular permeability, the cell cycle, and the leukocyte transmigration across endothelial wall.

To further elucidate the function and the interplay of the three molecules, we used a molecular approach. To this end, we constructed chimeric molecules consisting of Flag-tag or EGFP sequences fused to a soluble or a membrane bound form of CRAM-1, CRAM-2 or JAM (**summarized in Annex II**). When transfected into cell lines, the EGFP fusion products of CRAM-1 and JAM localized in cell-cell contacts, confirming a possible role of these molecules in the cell-cell communication. In contrast, CRAM-2 was more widely distributed on the cell surface. Moreover, the soluble construct of CRAM-1 blocked transendothelial migration of leukocytes in vitro, whereas soluble JAM showed only marginal effect. Altogether, our results suggest a central role of this new subfamily of adhesion molecules in the maintenance of vascular integrity and the function of the endothelial layer. Understanding the interplay between CRAM-1, JAM and CRAM-2 and their regulation will improve our knowledge of the relationship between the angiogenic switch, vascular permeability, inflammatory response and leukocyte transmigration. It will lead to new therapeutical interventions by using peptides or antibodies interfering with the JAM or the CRAM dependent physiology of the vascular endothelium. Counter acting the chronic inflammation and tumor development with reagents based on CRAM will be the main goals.

Materials and Methods

Cell lines

The thymic, embryonic, and brain derived endothelioma cell lines t-end.1, e-end.2, and b-end.5 were provided by Dr. W. Risau (Max Planc Institute, Bad Nauheim, Germany). The SV40 transformed Lymph node endothelial cell line TME, the adenovirus 5 DNA transformed human kidney epithelial cell lines 293 T and Bosc 23 were from Pr. B. Imhof (CMU, Geneva, Switzerland), the Thymic epithelium-derived cell line MTE4 from Pr P. Naquet (Centre d'Immunologie, Marseille-Luminy, France), the highly metastatic melanoma cell line B16 F10 from Dr. G. Nickolson (Houston, TX, USA). The squamous cell carcinoma KLN 205, teratocarcinoma NullI-SCC1, submandibular gland carcinoma SCA-9 and testis Leydig TM3 cells were obtained from the American Type Tissue Culture Collection (ATCC). Cells were grown in DMEM (Gibco BRL, Paisley, Scotland), supplemented with 10% FCS (PAA Laboratories, Linz, Austria), 2 mM Glutamine, 100 U/ml Penicillin and 100 µg/ml Streptomycin (all Gibco BRL). Adherent cells were detached by washing with PBS/ 0.15 mM EDTA followed by 5 min incubation in trypsin/EDTA at 37°C.

Co-culture Experiment

For co-culture experiments, $5x10^5$ t-end.1 cells were grown together with $2.5x10^4$ B16 F10 melanoma cells for 64 hours in 10 cm tissue culture dishes. As control, $5x10^5$ t-end.1 and $2.5x10^5$ B16 F10 cells were grown separately under the same conditions. Cells were then harvested and total RNA was directly extracted using Trizol reagent following manufacturer's instructions (Gibco BRL, Paisley, Scotland). Briefly, 3 ml Trizol was added to each tissue culture dish and then cells were lysed by vigorous pipetting. Lysates were incubated for 5 min at room temperature, 0.6 ml of chloroform was added and the mix was vigorously shaken for 15 sec. The aqueous phase was separated from organic phase by centrifugation (15 min, 3000 rpm, 4°C) and the RNA was recovered by isopropanol precipitation. Total RNA concentrations were determined by spectrophotometry (Ultrospec 4050, Biochrom, Cambridge, UK).

Targeted Differential Display: TDD.

cDNA was prepared from 5 µg total RNA from cells of co-culture experiments, employing oligo-dT (16-mer) primer and Superscript Reverse Transcriptase (Gibco BRL, Paisley, Scotland). For PCR amplification six sets of partially degenerated primers were designed based on the amino acid sequence Y(Y/Q/R)CXAS present in immunoglobulin C_2 type domains (see Fig 1). These primers allowed amplification of discrete cDNA. Some of the resulting PCR products (around 20%) corresponded to sequences coding for Ig superfamily members. The differential display was carried out as previously described

(Liang, 1992 and Pardee, 1995). For PCR P^{33}-ATP and Goldstar DNA-polymerase (Eurogentec, Seraing, Belgium) were used. The PCR conditions were calibrated to determine the minimum and maximum annealing temperatures of the degenerated primers. The parameters for the PCR were as follow: 45 sec at 94°C, 90 sec at 50°C, and 45 sec at 72°C repeated 40 times. Formamide/EDTA loading buffer was added and samples were denatured for 2 min at 94°C. The PCR products were separated on a 6% polyacrylamide gel, and autoradiographied using Kodak OM-Mat film. The band intensities were compared. Differentially expressed bands were cut out from the dried polyacrylamide gel and fragments were retrieved by boiling and ethanol precipitation as previously described (Liang et al, 1993). The PCR products were then reamplified using increased concentrations of dNTPs (0.2 mM instead of 2 μM) without P^{33}-ATP. The product of re-amplification was cloned into pGem-T Easy Vector (Promega Corp., Madison, WI, USA).

Cloning and Sequence Analysis

Competent DH5α E.coli were transformed with calcium chloride as described (Maniatis et al. 1989). Colonies were screened by PCR with T7 and Sp6 primers. Following 5 min denaturation at 95°C, parameters for PCR were: 30 sec at 95°C, 30 sec at 50°C, and 30 sec at 72°C, repeated 25 times. Positive clones were grown overnight at 37°C in 3 ml LB medium containing 100 μg/ml Ampicilin. Plasmid DNA was extracted using the Qiagen Miniprep Kit (Qiagen, Zurich, CH) and the nucleic acid sequences of two independent clones were determined using the Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech, UK) and the LI-COR DNA Analysis System (MWG-Biotech, Germany). Further sequence analysis and comparison were performed via the applications available on the ExPASy Molecular Biology Server i.e. Blast, Prosite, Swiss-Prot. Three different ESTs homologous to CRAM-1 were identified (Accession No. AA726206, AA052463 and AA175925). None of them encoded for a full length transcript and comprised the initiating ATG sequence. Therefore, the 5' coding sequence was obtained using the 5'RACE-PCR System for Rapid Amplification of cDNA Ends, Version 2.0 according to manu-facturer's instructions (Gibco BRL, Paisley, Scotland). The three primers used were designed based on the EST sequences as follows: $^{5'}$-GAGGTACTTGCATGTGCT-$^{3'}$ for synthesis of the first strand, $^{5'}$-CGACAGGTGTCAGATAACA-$^{3'}$ and $^{5'}$-CACCCTCCTCACTCGT-$^{3'}$ for the two nested PCRs. The 5'RACE-PCR product was cloned into pGem-T Vector. To obtain the full length coding sequence for CRAM-1, the cloned 5'RACE-PCR product and the EST (accession No. AA726206) were digested with HpaI and NotI restriction enzymes and ligated into pGem-t vector.

Cloning of full length CRAM-2 was based on the same strategy of sequence comparison and 5'Race technic. The full-length cDNA encoding CRAM-2 was finally obtained from

ESTs accession numbers: AA690843 and W80145. These two clones differ by the length of the 3' untranslated region. The cDNA encoding JAM was kindly provided by Pr Ph.Naquet (CIML, Marseille-Luminy).

Northern blot

The size of transcript was determined by Northern blot analysis as previously described (McMaster and Carmichael, 1977). Total mRNA from cells or tissues were extracted using Trizol (Life technology) according to instructions from the manufacturer. Poly-A mRNA was extracted from 250 µg total RNA with the Oligotex mRNA Purification Kit (Qiagen, Zurich, CH) and run on agarose gel following glyoxal denaturation. The RNAs were transferred overnight in 20xSSC (3 M NaCl, 0.3M Na-citrate) onto Hybond-N nylon membrane (Amersham Pharmacia Biotech, UK) and crosslinked with UV Stratalinker 1800 (Stratagene, La Jolla, USA) at 120 mJ/cm^2. The riboprobes were prepared from the cloned soluble molecules corresponding to the first domain and hybridized overnight at 62°C in a hybridization mix containing 50% formamide. The β-actin probe was used as internal control.

Semiquantitative PCR

The presence and relative amount of transcript in various tissues and cell lines was determined by Semiquantitative Polymerase Chain Reaction (Ruiz et al. 1997). Total RNA and cDNA corresponding to cell lines were prepared as described above. The number of PCR cycles (22, 25 and 30) were calibrated to observe amplification signals below saturation levels. The cycles consisted of denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, and elongation at 72°C for 45 sec followed by terminal elongation at 72°C for 2 min. As internal control, the following primers specific for Hprt cDNA were used to amplify a 350 bp long fragment (Foss et al. 1998): $^{5'}$-GTTGGATACAGGCCAGACTTTGTTG-$^{3'}$ and $^{5'}$-GAGGGTAGGCTGGCCTATAGGCT-$^{3'}$. For the detection of the CRAM-1 transcript the $^{5'}$-GACTCACAGACAAGTGAC-$^{3'}$ and $^{5'}$-CACCCTCCTCACTCGT-$^{3'}$ primer pair was chosen to result in a 750 bp long amplification product. For the detection of the CRAM-2 transcript the primer pair was chosen to result

Confluency experiment

The effect of endothelial cell confluency on CRAM-1 mRNA levels was investigated. Additionally, since B16 showed endogenous CRAM-1 expression, we used the KLN 205 squamous cell carcinoma as tumor cell line to confirm the regulation of CRAM-1 mRNA in endothelial cells upon coculture experiment. Two cell lines, t-end.1 and TME, were grown separately and in combination with KLN 205 cells in 6, 10 and 15 cm diameter tissue culture dishes as described above. The initial number of cells was set at 2x10^5 for t-

end.1 and TME, and at $1x10^4$ for KLN 205 cells, to result in a series of comparable confluency levels in the range of 10-100%. The relative quantities of transcript were determined by PCR and visualized on 1.2% agarose gel. Quantification and analysis of band intensities was done using RFLPscan Plus software (Scanalytics, CSPI, Billerica, MA, USA).

Flag-tag and EGFP constructs

The mammalian expression vector pcDNA 3 (Invitrogen, Leek, Holland) was modified by integrating the Flag-Tag (G. Wiedle, Dep. of Pathology, CMU, Geneva) or EGFP (C. Ballestrem) coding sequences. Flag-Tag constructs containing coding sequences for the soluble forms of the JAM, CRAM-1 and CRAM-2 proteins were prepared by PCR. In all cases, the forward primers were designed to fit ATG initiation region and are named: LeadJAM, LeadCRAM-1 and LeadCRAM-2 respectively. The reverse primers were designed in the sequences encoding the hinge region for the one Ig soluble form (primers Rev1do in the list below) or in the sequence encoding the region between the C2 and transmembrane domains for two Ig domains soluble molecules (primers Rev2do in the list below). All reverse primers had 3' extensions containing a XbaI restriction site for direct in frame cloning in the Flag-tag modified vector. Pfu DNA polymerase was employed in the PCR to avoid frequent mutations (Stratagen, La Jolla, CA, USA). The PCR fragments were then digested with XbaI and cloned into the pcDNA-3 Flag-Tag vector, digested by EcoRI, filled by klenow and followed by an XbaI digest. All constructs were then sequenced and analysed using the LI-COR system.

For EGFP constructs, a different strategy was used. Cloning of CRAM-1 and JAM consisted in using the 3'HpaI or 3'ScaI respectively and the 5' EcoRI sites in order to clone coding sequence for CRAM-1 and JAM in frame with the sequence encoding EGFP. Since no equivalent restriction sites were available in the sequence of CRAM-2, we used a PCR approach to obtain the CRAM-2-EGFP chimeric molecule. LeadCRAM-2 primer was used as forward primer in combination with the reverse primer: RevAgeCRAM-2.

LeadJAM:	5'-GTAACTGTAATGGGCACCGAG-3'
LeadCRAM-1:	5'-TCGACATGGCGCTGAGC-3'
LeadCRAM-2:	5'-TCAGCTAGGCAGCCAGCT-3'
Rev1doJAM:	5'-GCTCTAGAACTGATCGTCGGCTTGGA-3'
Rev1doCRAM-1:	5'-GCTCTAGACAGTGTTGCCGTCTTGCCTACAG-3'
Rev1doCRAM-2:	5'-GCTCTAGAAGGAACAGCAGGAGCCACTAG-3'
Rev2doJAM:	5'-GCTCTAGACACAGCATCCATGTGTGCAGCCTC-3'
Rev2doCRAM-1:	5'-GCTCTAGAATAGACTTCCATGTCCTGCC-3'

Rev2doCRAM-2: $^{5'}$GCTCTAGAATCTACTTGCATTCGCTTCC-$^{3'}$
RevAgeCRAM-2: $^{5'}$CGACCGGTGTGACTTTAGATGCAGGACTGCC-$^{3'}$

Transfection and purification of s luble m lecules

Transient transfection of 293 T, Bosc 23 or stable transfection of CHO cells with the soluble Ig1Do and Ig2Do Flagtag/pcDNA-3 constructs were carried out using Lipofectamine Reagent according to manufacturer's instructions (Gibco BRL, Paisley, Scotland). Following transfection supernatants were collected every two days during a ten days period. M2 Beads (Kodak, New Haven, USA) covalently linked to anti-Flag antibody were washed twice with PBS containing a Protease Inhibitor Mix (Boehringer Mannheim, Germany), The beads were then incubated at 4°C for 3 hours with supernatant from the transfected cells. After five washes with PBS containing protease inhibitors, a column was packed with the beads, and recombinant molecules were eluted with 10mM glycine buffer pH 3,4 according to the manufacturer. The eluted fractions containing the recombinant proteins were then concentrated on Centricon-10 (Millipore) and dialysed against PBS. Final protein concentration was determined using the Micro BCA assay (Biorad). The purified products were then submitted to a polyacrylamide SDS gel electrophoresis followed by coomassie blue staining to analyse their purity.

Transmigration assays

Leukocyte transmigration across endothelial cells was performed as previously described (Wheerasinghe et al.). Briefly, $1x10^5$ t-end cells were cultured for two days in transwell units (polycarbonate filters, 5µm pore size, costar) in the presence of 1µg of Ig soluble recombinant molecules: sJAM 2do or sCRAM-1 2do. After two days, $1x10^6$ leukocytes obtained from lymph nodes and Peyer's patches were added to the upper compartment, and the number of transmigrated cell was monitored during the experiment every hour. After 4 hours, transmigrated cells obtained from five independent wells were pooled and submitted to cytofluorimetric analysis for B-and T-cell markers B220 and CD3. Results were obtained using a Facscalibur machine and the Cell-Quest analysis program (Becton-Dickinson).

Results

Targeted Differential Display

The regulation of genes in endothelial cells depends on their environment. We aimed to identify genes that undergo regulation upon the contact of endothelium with tumor cells. For this purpose, we developed an in vitro assay using the coculture of melanoma tumor cells (B16) with an endothelioma cell line (t-end). Total RNA extracted from the mix was used as template to prepare cDNA submitted to a differential PCR screen. The cDNA obtained from the endothelial or melanoma cells cultured on their own were used as controls. We compared the three different patterns to identify the transcripts regulated by the coculture condition. To limit the analysis to the sequences encoding for cell surface molecules of Ig superfamily, we used partially degenerated primers that target the sequence surrounding the C-terminal cysteine of C2 domains in Ig superfamily molecules. The most reproducible pattern of PCR products was obtained using primers that encode the sequence YYCxAS1 (Fig 1A). We named this improved method of RNA display technique TDD for "Targeted Differential Display". In repeated experiments of TDD, sixteen differentially expressed genes were identified. Following cloning, nucleotide and amino acid sequence analysis, three of the sixteen PCR products were possible candidates encoding for unknown members of the Ig superfamily. One of the

three candidates (CRAM-1) was chosen for further investigation. When grown separately t-end.1 endothelial and B16 melanoma cells expressed high levels of the CRAM-1 transcript. However, under coculture conditions the level of CRAM-1 expression was down regulated (Fig 1B). Translation of a 350 bp long fragment corresponding to CRAM-1 showed the amino acid sequence YYCxAS indicating the endings of an Ig C_2 domain followed by an open reading frame (ORF) containing an hydrophobic stretch of 18 amino-acids that signed a transmembrane region.

CRAM-1, a member of the Immunoglobulin Superfamily

Sequence comparison between the PCR product sequence and nucleotide databases revealed homologous and identical sequences in mouse ESTs databases. The presence of ESTs indicated that the PCR product corresponded to a sequence expressed in vivo. Three ESTs were found to contain a 300bp long sequence at their 5' end, which was identical to 300 bp in the TDD product. The 3' ends of each EST contained a poly-A tail. In total the ESTs were 1270 bp in length and corresponded to the 3'end of the CRAM transcript. Since the 5' end of the transcript was missing in the EST cDNA clone, it was obtained by 5'RACE-PCR. The resulting 1980 nucleotide long full length coding sequence of the postulated CRAM-1 cDNA is shown in Fig 2A. There was a strong consensus site (GACATGG) for translation initiation (Kozak M.1989), 16 bp downstream from the 5' end, followed by a single ORF predicting a protein of 310 amino

acid. The 31 amino-acid region subsequent to the potential initiating methionine, was characteristic of a signal peptide. The cleavage site was predicted to be at Ala 31-Val 32 (Nielsen et al. 1997). The putative structure of the murine CRAM-1 protein is shown in Fig 2B and consists of an extracellular region with a variable heavy chain and a constant type 2 like immunoglobulin domain (Pfam, The Sanger Centre and Blast) with two potential N-linked glycosylation sites (aa 104 and 192). The hydrophobicity analysis (Tmpred, ISREC) predicted a transmembrane region between positions 242-260. The postulated cytoplasmic domain consisted of 49 amino acids and contained a number of highly conserved Ser/Thr and Tyr phosphorylation sites (Fig 2A, residues in italic). The search of known patterns with the Prosite program identified the motifs SSK/SYK as protein kinase C, SKQD/TSEE as CK2 and KQDGESY/KHDGVNY as Tyrosine kinase phosphorylation signatures (Woodgett et al.1986, Pinna 1990 and Hunter 1982).

JAM, CRAM-1 and CRAM-2 define a new subfamily

Several proteins showed high homology to CRAM-1. Two members of the Ig Superfamily: Human A33 antigen and part of the mouse neural cell adhesion molecule, N-CAM were found to have 41% and 46% homology with CRAM-1 respectively. JAM, another member of the Ig Sf, had a similar structure as CRAM-1 with 34% amino acid sequence identity, and 54% homology (Martin-Padura et al. 1998). We used the significant identity between JAM and CRAM-1 to find a third closely related sequence in

EST databases, namely CRAM-2. The identity between the three molecules suggested the existence of a new subfamily of molecules in the Ig superfamily (Fig 3). The homology concerned not only the overall structure of V and C2 domains (C_{54} to C_{118} and C_{147} to C_{235} in Fig 3) but also sequences inside the cytoplasmic domains. Interestingly, the most divergent regions between the three molecules were found at the beginning of the V domain (position 40 to 60) and in the proximal cytoplasmic part (position 280 to 300). The functions of these two regions correspond to sequences involved in ligand binding and signal transduction in other members of the Ig superfamily suggesting a role of JAM, CRAM-1 and CRAM-2 in cell-cell communication.

Tissue Distribution of JAM, CRAM-1 and CRAM-2 mRNA

Expression of the three transcripts in cells of different origin was detected using RT-PCR. All endothelial, epithelial and most tumor cell lines tested, were positive, although at varying degrees for the different transcripts (Fig 4A). The highest expression level for CRAM-1 was found in the SV40 transformed HEV cell line TME (lane 9), and in the embryonic endothelial cell line e-end 2 (lane 4). The CRAM-2 and JAM transcripts showed a more restricted distribution, and were found in adult endothelial cell lines together with the CRAM-1 transcript (lanes 3, 7, 9 and 12). Notably, JAM and CRAM-2 transcripts were strongly down regulated by TNF treatment of endothelial cells whereas the level of CRAM-1 transcript remained unchanged (lanes 2 and 11). Interestingly, an

embryonic endothelial cell line (lane 4) or an adult endothelial cell line representing an angiogenic variant of t-end (lane 6, manuscript in preparation) failed to express JAM or CRAM-2.

To confirm these RT-PCR data, we performed northern blot analysis of CRAM-1 and JAM transcripts (Fig 4B). JAM was previously described to be highly expressed in lungs and heart and poorly expressed in spleen and brain. Here, we found additional high expression of JAM in thymus, Peyer's patches and Lymph nodes. Interestingly, CRAM-1 transcripts showed similar tissue distribution to JAM in adult lymphoid organs. Furthermore, CRAM-1 was well expressed in intestine, which contained numerous lymphoid structures, and its expression in other adult tissues was rather low (not shown). According to the high expression of CRAM-1 found in the embryonic endothelial cell line e-end.2, we detected the transcript encoding CRAM-1 during embryogenesis as early as day 7 (Fig 4B, lane E7). These results suggest that CRAM-1 is widely expressed during embryogenesis and shows a restricted expression to epithelial or endothelial compartments in adult tissues. This is in agreement with the idea that it may play a role in the establishment and the maintenance of the polarized organization of cells.

JAM and CRAM-1 support homotypic interactions

To further test how JAM, CRAM-1 or CRAM-2 may play a role in polarized endothelial or epithelial cells, we transfected CHO cells with EGFP chimeric molecules (for details

see annex II). The JAM construct localized remarkably well to cell-cell contacts as previously described by classical immuno-histochemistry (Fig 5A, a). This proved that the EGFP did not perturb the correct function of the chimeric molecule. Interestingly, CRAM-1-EGFP showed a similar distribution (Fig 5A, b). It was noteworthy that CRAM-1-EGFP localized to cell-cell contacts when both neighbors were transfected cells, and was diffusely distributed when one of the cells did not express the construct (Fig 5A, arrows). This suggests that CRAM-1 interacted in a homophilic way. Moreover, the homotypic interaction could not be disrupted by incubation with 5mM EGTA suggesting a calcium independent interaction (not shown). The integrity of the chimeric molecules was confirmed by immunoprecipitation. As shown in Fig 5B, the JAM-EGFP and the CRAM-1-EGFP proteins are correctly expressed with their predicted molecular weights under non-reducing conditions. A more refined analysis of the EGFP location is depicted in Fig 5C showing that CRAM-2-EGFP strongly differed from JAM-EGFP and CRAM-1-EGFP. The three dimensional reconstitution showed an exclusive location of JAM and CRAM-1 constructs in cell-cell contacts whereas CRAM-2-EGFP was evenly distributed on the cell surface with a preferential targeting to microspikes. This indicated that CRAM-2 may act via heterotypic interactions and may not play a role in the maintenance of monolayer integrity as it is the case for JAM and CRAM-1. Another possibility is that the ligand for CRAM-2 is not present on CHO cell surface but is expressed by endothelial cells.

Tightness and leukocyte migration.

Finally, in order to understand how molecules influence the integrity of the endothelial cell monolayer and how they regulate the function of the vascular endothelium, we performed leukocyte trans-endothelial migration assays in the presence of recombinant soluble JAM or CRAM-1. Endothelial cells were cultured for two days in the presence of sJAM-Ig2Do or sCRAM-1-Ig2Do. The monolayer integrity was not affected during this period, probably due to the molecular redundancy of the mechanism of cell-cell contact formation. The transmigration assay was performed in the presence of 1μg of recombinant soluble molecules. As shown in Fig 6A, the number of transmigrating cells was poorly affected by the presence of sJAM-Ig2Do (open squares) during the first three hours. After four hours, the number of transmigrated cells increased when compared to the control (dashed line). In contrast, the presence of sCRAM-1-Ig2Do (closed circles) strongly reduced the number of transmigrating cells. Since the leukocyte populations were heterogeneous, we evaluated if sCRAM-1-Ig2Do acted on a specific leukocyte subpopulation or whether transmigration was blocked without specificity. For this purpose, we labeled the transmigrated cells for the lymphocyte markers CD3 and B220 (Fig 6B). Remarkably, sCRAM-1-Ig2Do specifically blocked the transmigration of non lymphoid leukocytes, i.e. myeloid lineage cells (central panel, dashed columns). In contrast, sJAM-Ig2Do poorly increased the number of transmigrating T cells (left panel, white column) without any effect on other cell subpopulations. These results led us to

hypothesize that the engagement of JAM or CRAM-1 between endothelial cells may regulate the function of the endothelial layer. It could be expected that the molecules of this family will become a barrier when endothelial cells reach confluency. To this end, we explored the regulation of CRAM-1 transcripts in endothelial cells under different culture conditions.

CRAM is down regulated by high confluency

Since the transcript that encodes CRAM-1 is not regulated by TNF, but is downregulated when the endothelium was cocultured with tumor cells, we used a confluency assay to further explore this regulation. Under low confluency, the cells were actively cycling and CRAM-1 interactions did not occur whereas under high confluency the cells divided less and CRAM-1 was engaged. We determined the level of CRAM-1 mRNA expression under various cell densities by semi-quantitative RT-PCR. As shown in Fig 7, the expression level of CRAM-1 transcripts decreased when confluency was reached (lanes 1, 2, 3 in Fig. 7 correspond to 100, 50, and 10% confluency respectively). This effect was hardly detectable with the t-end cells but was more pronounced with the TME cell line which highly expressed CRAM-1. The down regulation of CRAM-1 in endothelia was also enhanced when the endothelial cells were co-cultured with KLN 205 carcinoma cells which themselves do not express CRAM-1. This confirmed the link between CRAM-1 expression and the cell cycle since tumor cells were described to increase the growth rate of endothelial cells upon contact. It is noteworthy that the results obtained with KLN 205

carcinoma cells was identical to the one used in our original screening strategy with the B16 melanoma tumor. This suggested a general mechanism by which tumors affect endothelial behavior. Further work will lead to the understanding of the mechanism of regulation of CRAM-1 and its role in tumor development.

Discussion

This paper reports the use of a new screening strategy to identify regulated transcripts encoding members of the Ig superfamily of adhesion molecules. We describe here the cloning with this method of the new molecule CRAM-1 as a regulated transcript. The regulation observed in endothelial cells grown in the presence of tumors is confirmed by semi quantitative RT-PCR and is shown to be dependant on the growth phase of the cells. Due to differential expression under changing cell confluency conditions, the name CRAM-1 for "Confluency Regulated Adhesion Molecule-1", is proposed. We also describe a closely related sequence to CRAM-1 named CRAM-2. CRAM-1 and –2 represent the prototypes of a new subfamily of adhesion molecules which also includes the recently described molecule JAM. CRAM-1 and JAM are preferentially expressed by endothelial and epithelial tissue at the cell-cell contacts and confer special properties to polarized layer. The effect of recombinant soluble molecules in a transendothelial migration assay and the regulation of JAM, CRAM-1 and CRAM-2 suggest that these three molecules play an important role in the maintenance of vascular physiology.

The new screening strategy, namely Targeted Differential Display (TDD), has proved to be an efficient technique in selectively amplifying cDNA of interest. TDD successfully exploited the use of partially degenerated primers to confer selective targeting to the conserved region, Y(Y/Q/R)CXAS, of C_2 like Ig domains. Repeated experiments lead to

reproducible display patterns. Out of 16 differentially expressed transcripts, three correspond to genes with significant homology to conserved Ig sequences. This increase in specificity manages to overcome the major difficulties in the already known techniques of classical RNA fingerprinting and differential display. RNA fingerprinting has long been used for the identification of differentially expressed genes. However, due to the sequence specific primers employed, this method detects only the transcripts of selected and already known proteins (Dube 1973). On the other hand, RNA display employs random primers and involves the non-specific amplification of transcripts. The aim in this case is to pinpoint any differences in mRNA levels between two biological systems, which are submitted to comparison (Shima et al. 1995). TDD is an advanced screening method that combines the specificity of RNA fingerprinting with the degeneracy of Differential RNA Display resulting in selectivity. Due to the targeting of related transcripts, this technique significantly reduced the time needed for screening. The identification of new members of specific protein families, therefore, becomes possible. This is a substantial improvement of the reported non-specific screening strategies.

Out of the three regulated transcripts, coding for putative Ig Sf molecules, one was chosen for further investigation. The availability of ESTs provided a good starting point toward obtaining of the full-length sequence of the corresponding cDNA. The ESTs are identical, apart from a missing 310 bp segment in one of them. Considering that the slightly shorter EST was obtained from a mouse fetal library, it is possible that the

missing sequence has a regulatory function during development. In fact, the control of protein expression by regulatory elements present in the 3'untranslated region of poly A^+mRNA is well documented. Post-transcriptional regulation of VEGF mRNA relies on specific sequence motifs that allow regulatory proteins to bind within its 3'UTR. These binding proteins have opposite functions: one that increases and one that decreases the stability of the transcript (Levy et al.1996). This type of control is also shown to affect the half-life of the LDL receptor mRNA (Wilson et al. 1998). The 310 bp long segment missing from the shorter, fetal EST constitutes a significant part of the 1 kb long total 3'UTR. The possibility arises, that this region contains binding motifs for regulatory proteins that decrease the half life of the CRAM-1 transcript.

Translation of the sequence of the PCR product obtained by TDD results in an open reading frame. The obtained amino acid sequence starts with the YYC*AS motif, the initially targeted C_2 domain signature. The presence of a stop codon is not a determining factor, since some miss priming is expected due to the use of partially degenerated primers. The following ORF contains a stretch of 18 hydrophobic amino acids as a putative transmembrane region. This finding is very important as it increased the possibility of identifying a cell surface protein, that is one of the criteria for adhesion molecules. The 1980 bp long full length cDNA is assembled from the EST and the 5'RACE product and translated into an extended ORF containing an initiating Met near

its N-terminal. Two immunoglobulin domains, V_H and C_2, and a transmembrane region follow the postulated starting site.

Similarity searches based on the amino acid sequence of CRAM-1 identified several ESTs and the adhesion molecule JAM as homologous. One of the ESTs encoded for the protein that we identified as CRAM-2. The structural features of the three proteins are the same and allowed the description of a new subfamily of adhesion molecules among the Ig Sf comprising one V like and one C2 Ig domain. JAM is a recently reported member of the immunoglobulin superfamily that distributes at intercellular junctions and could modulate monocyte transmigration (Martin-Padura et al. 1998). It is noteworthy that JAM was originally described as an Ig molecule comprising two V like domains due to the lack of the second disulfide bridge in the C2 domain. Nevertheless, the identity between JAM, CRAM-1 and CRAM-2 extending over the structurally important residues, and amino acids within the hinge region or the cytoplasmic part, allow to classify them in the same subfamily. We used these common features to construct recombinant proteins in order to study the functions of JAM, CRAM-1 and CRAM-2. In the present paper, effects of sJAM-Ig2Do and sCRAM-1-Ig2Do are described in an in vitro transmigration assay. Specific blocking effects on the migration of myeloid cells could be observed with sCRAM-1-Ig2Do whereas sJAM-Ig2Do showed only a small effect on lymphocytes. Work is in progress to identify the binding sites of each molecule that interfered in this assay.

At present, we may hypothesize that JAM and CRAM-1 may have similar but distinctive functions due to their common features. CRAM-2 that did not show homotypic interactions may act by a slightly different mechanism. Ongoing experiments will demonstrate the respective roles of JAM, CRAM-1 and CRAM-2 in the vascular functions.

Interestingly, JAM and CRAM-2 transcripts showed a similar tissue d istribution and regulation of expression under the influence of TNF, suggesting that they act by similar physiological mechanisms. In contrast, CRAM-1 transcripts are not regulated by TNF but rather by the rate of proliferation or the density of endotheli al cells. In fact, over expression of CRAM-1 transcripts in cycling cells and its down regulation in quiescent cells suggest that this molecule participates in the establishment of a continuous monolayer. Its function in confluent monolayers of cells may be the maintenance of the endothelial cell layer and the related properties. The loss of gene regulation induced by TNF is interesting and remains to be explained in models of chronic inflammation. Since different leukocyte populations have to migrate to the site of immune response, we suggest that non lymphoid cells may migrate via a CRAM-1 dependant mechanism, whereas lymphoid cells may migrate via JAM or CRAM-2. In this case we could modulate the immune response by using combinations of different soluble recombinant molecules.

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Claims

1. The involvement of CRAM-1 and 2 in angiogenesis and its inhibition with peptides recombinant proteins and/or antibodies based on the sequence of the two CRAMs.

2. The involvement of CRAM-1 and 2 in inflammatory reactions of the vascular endothelium and its blocking with peptides recombinant proteins and/or antibodies based on the sequence of the two CRAMs.

3. The involvement of CRAM-1 and 2 in vascular permeability and its modulation with peptides recombinant proteins and/or antibodies based on the sequence of the two CRAMs.

4. The involvement of CRAM-1 and 2 in leukocyte transmigration and its blocking with peptides recombinant proteins and/or antibodies based on the sequence of the two CRAMs.

5. The involvement of CRAM-1 or 2 in vascular homeostasy and its reconstitution with peptides, recombinant proteins and/or antibodies based on the sequence of the two CRAMs.

6. The use of DNA probes, recombinant proteins and antibodies based on CRAM-1 and 2 as tumor markers.

>muCRAM-1

CAGACATTCCCCTCGACATGGCGCTGAGCCGGCGGCTGCGACTTCGACTGtACGCGCGGCTGCCTCACTTCTTCCTGCTG
CTGCTCTTCAGGGGCTGCATGATAGAGGCAGTGAATCTCAAATCCAGCAACCGAAACCCAGTGGTACATGAATTTGAAAG
TGTGGAATTGTCTTGCATCATTACGCACTCACAGACAAGTGACCCTAGGATTGAATGGAAGAAAATCCAAGATGGCCAAA
CCACATATGTGTATTTTGACAACAAGATTCAAGGAGACCTGGCAGGTCGCACAGATGTGTTTGGAAAAACTTCCCTGAGG
ATCTGGAATGTGACACGATCGGATTCAGCCATCTATCGCTGTGAGGTCGTTGCTCTAAATGACCGAAAAGAAGTTGATGA
GATTACCATTGAGTTAATTGTGCAAGTGAAGCCAGTGACCCCTGTCTGCAGAATTCCAGCCGCTGTACCTGTAGGCAAGA
CGGCAACACTGCAGTGCCAAGAGAGCGAGGGCTATCCCCGGCCTCACTACAGCTGGTACCGCAATGATGTGCCACTGCCT
ACAGATTCCAGAGCCAATCCCAGGTTCCAGAATTCCTCTTTCCATGTGAACTCGGAGACAGGCACTCTGGTTTTCAATGC
TGTCCACAAGGACGACTCTGGGCAGTACTACTGCATTGCTTCCAATGACGCAGGTGCAGCCAGGTGTGAGGGGCAGGACA
TGGAAGTCTATGATTTGAACATTGCTGGGATTATTGGGGGAGTCCTTGTTGTCCTTATTGTTCTTGCTGTGATTACGATG
GGCATCTGCTGTGCGTACAGACGAGGCTGCTTCATCAGCAGTAAACAAGATGGAGAAAGCTATAAGAGCCCAGGGAAGCA
TGACGGTGTTAACTACATCCGGACGAGTGAGGAGGGTGACTTCAGACACAAATCGTCCTTTGTTATCTGACACCTGTCGG
CTGGGAGAGCACATGCAAGTACCTCTGTTGGAAGCTGGTCACAGGGCTGCTGTGAGCCCAGAGCTCCTGACAAAGCCACC
CGGGCAGAAGCTTTTTGTTTTGGCCAAAGTTGATGACTCCTTCCTTCCTTCCTTCCTCTTTAACAAGCCACAAGAATAAa
AGGAAGCCTCCTGAAGATGGATGTAGACACAGATTGTTGCTAGCCTGACCTCATTATGGGGATTAGGGTGATCTTCAAGG
CCTTTCTGGTCTCCGTTCTCCCATGCAGGGCAATTTGGACTGTTTTTGCCCCAGGCTGTTTAGCTGCCAGGACAACACTG
GCAGAGAGAGGCTGAGGCGCTGGGCTGCAGTAGCAGCAGGCAACAGCCTGATGCCTGTGACAGTGCCCCAGGAAGGTTTT
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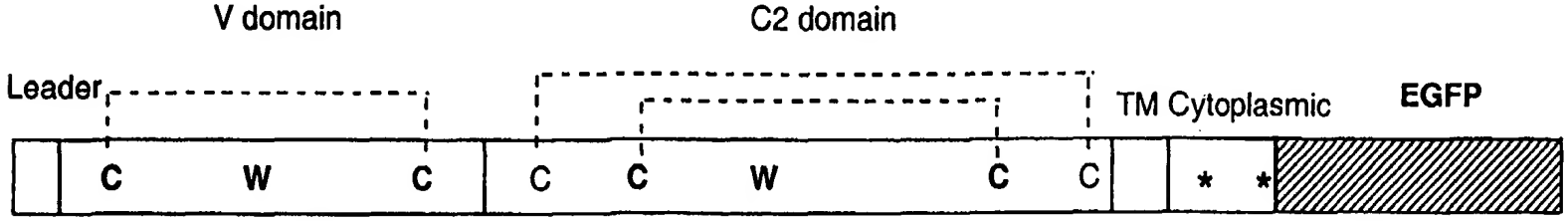
TATATAAACTGTAAATATATTGCCATACAGTGTTTCAAAACGTATTTTTTTATAATGAGTTCAACTTAAGGTAGAAGGCT
TGGGCTGCTAGTGTTTAATTGGAAAATACCAGTAGTAAAGTCTTTTAAGGAGTTTTCTTAAGGAGGCTGGCTGAATATTC
CTTTGTTCAAAAGAAGTTTTAGCATTTTTCATAAGAAAACTTACTCTGTCTGACCACTGTTGCTTAGGAAACCATTAAAG
AATTCCAATCTAAaaaaaaaaaa

>muCRAM-2

CAGAaTTCGGCACGAgGGTCTGGgGGCGGGGGGCCGACCTACGGgTtCTCCCTCAAGAGCTAATCTCTGCcGCCACTCGC
TTAGgACCCTGCGGACACCGCGTCCCGcGTCCACgCCCTCCCCTCAACCCTCTTCCACCCTTCAAAAGAAGGACTGTCCA
GACACCACGTCCTAGGGCCAGAaGACCTGCCCCCACGACAGTcGCTGGAGACACCCCAGACCGGAGAGACTGACATCGGG
ACAGGACCCGCCCCTCTGCTTCCACCTCTCAGGGACCTCCTCTGCTCCGCCGCCGGGCGAAGTGCTGGGAGACCCAGCCG
CCTGTcGCGCTCCTGCAGGGGGACCCTCAGCTAGGCAGCCAGCTGGCGCCCGCGTAGATGGCGAGGAGCCCCCAAGGCCT
CCTGATGCTGCTGCTGCTACACTACTTGATCGTCGCCCTGGACTATCATAAGGCAAATGGGTTTTCTGCATCAAAAGACC
ACCGTCAAGAAGTCACAGTAATAGAGTTCCAAGAGGCTATTTTGGCTTGTAAAACCCCAAAGAAGACTACCTCCTCCAGA
CTGGAGTGGAAGAAGGTGGGACAGGGGGTCTCCTTGGTCTACTACCAACAGGCTCTCCAAGGTGACTTTAAAGACCGTGC
TGAGATGATAGATTTCAATATACGAATCAAAAATGTTACAAGAAGTGATGCTGGAGAGTATCGCTGTGAAGTCAGCGCTC
CGACTGAGCAAGGCCAGAACCTGCAGGAAGAtAAAGTcATGCTAGAAGTACTAGTGGCTCCTGCTGTTCCtGCCTGTGAA
GTGCCCACTTCTGTTATGACTGGAAGTGTGGTGGAGCTACGATGCCAGGATAAAGAAGGAAacCCAGCTCCGGAGTACAT
CTGGTTTAAAGATGGCACAAGTTTGCTAGGGAATCCAAAAGGCGGCACACACAACAACAGCtCGTACACAAATGAACACG
AaTCTGGAATTCTGCAATTCAACATGATTTCCAAGATGGACAGTGGAGAGTATTACTGCGAAGCCCGGAACTCTGTCGGA
CaCCGCAGGTGCCCTGGGAAGCGAATGCAAGTAGATGTTCTCAACATAAGCGGCATCATAGCAACGGTTGTGGTGGTGGC
CTTCGTGATTTCTGTaTGTGGCCTTGGCACATGCTATGCTCAGAGGAAAGGCTACTTTTCAAAAGAAACTTCCTTCCAGA
AGGGCAGTCCTGCATCTAAAGTCACTACGATGgGCGAAAATGATTTCAgGCACACAAAATCCTTTATAATTTAAAAGAAT
TCCAGTTTTGgGCTGCCCAAAACCAGTTGTCACATGTTATTAAAATATTGTAAAACTCTGTGTCTTACACTTGCAAAGTG
ATGAAGAAATATGAAAGGGGAGTTCATCAGAAGTTTTATGATCTCTAACTCACAAGAAATATTTTAAGCAAAACGTTCTT
GCCATCACTAAATTACAACCTGGCATCTTGTGTTGACCTAAAGGAAATGTCTGGTAATATTCTGGTTTTTGAAGGCAAAT
GAATGTCAGTTTGGAGTTGACTATATCACACTGACTGTAAGGCTAATCCAAGAAGCAAGAATATAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAATTTC

Annex I: murine cDNA sequences encoding the CRAM-1 and CRAM-2 proteins. muCRAM-1 was subcloned in pcDNA3 vector and sequenced using Sp6 and T7 primers. muCRAM-2 was obtained as IMAGE clone from EST library (Ac: AA690843 and W80145) and was sequenced in the pT7T3-DPac vector using T7 and T3 primers. Internal primers were used to confirm sequences at least twice on each cDNA strands.



JAM-EGFP QPS_{285}-EGFP

CRAM-1-EGFP DGV_{291}-EGFP

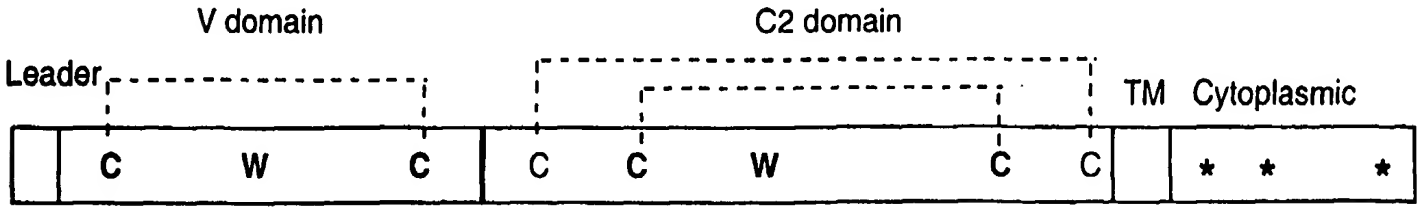
CRAM-2-EGFP KVT_{283}-EGFP

Tac-JAM Tac-W_{258}FAY....

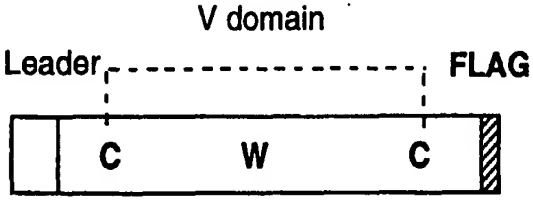
Tac-CRAM-1 Tac-C_{264}CAY....

Tac-CRAM-2 Tac-C_{259}YAQ....

AnnexII: Schematic representation of the molecular tools used in the study.
The structure and the important residues for of the new family are depicted in the upper top panel. The stars represent the putative phosphorylation sites in the cytoplasmic part of the three molecules. The second canonical Cys residue of the C2 domain is missing in the JAM sequence. Different chimeric molecules are represented below with the position and the surrounding residues of the fusion sites. Part of the molecules originating from JAM, CRAM-1 or CRAM-2 sequences are shown in white background.



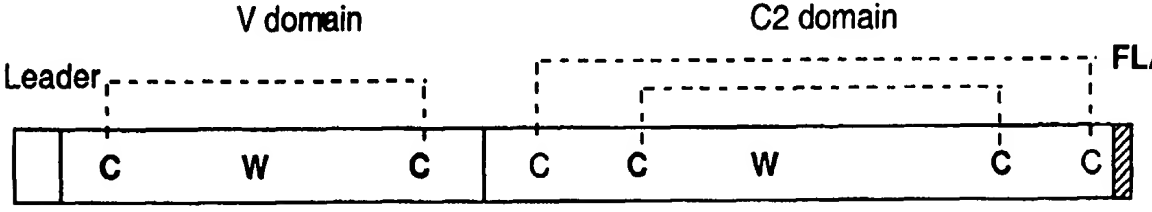
JAM	LTC_{49}	EWK_{62}	YTC_{108}		LTC_{152}	W_{164}	NSS_{187}	YYC_{212}		T_{268}	S_{285}	
CRAM-1	LSC_{53}	EWK_{68}	YRC_{115}	PVC_{144}	LQC_{160}	W_{173}	NSS_{194}	YYC_{219}	RC_{225}	S_{274}	S_{281}	S_{297}
CRAM-2	LAC_{51}	EWK_{65}	YRC_{110}	PAC_{140}	LRC_{156}	W_{169}	NSS_{189}	YYC_{214}	RC_{230}			T_{285}



sJAM-Ig1do ISV_{138}-FLAG

sCRAM-1-Ig1do TLQ_{159}-FLAG

sCRAM-2-Ig1do AVP_{138}-FLAG



sJAM-Ig2do AVE_{233}-FLAG

sCRAM-1-Ig2do EVY_{238}-FLAG

sCRAM-2-Ig2do QVD_{233}-FLAG

A

Y	R	C	X	A	S1
TAY	AGN	TGY	NNN	GCY	TCY AA

Y	R	C	X	A	S2
TAY	AGN	TGY	NNN	GCY	AGY AA

Y	Q	C	X	A	S1
TAY	CRG	TGY	NNN	GCY	TCY AA

Y	Q	C	X	A	S2
TAY	CRG	TGY	NNN	GCY	AGY AA

Y	Y	C	X	A	S1
TAY	TAY	TGY	NNN	GCY	TCY AA

Y	Y	C	X	A	S2
TAY	TAY	TGY	NNN	GCY	AGY AA

B

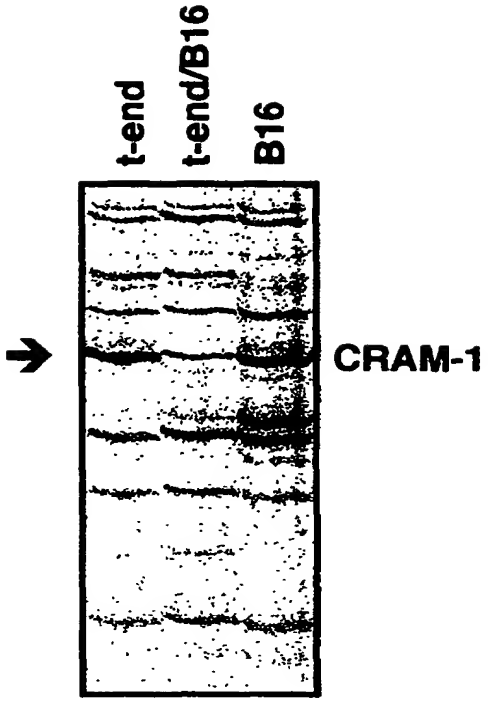


Fig 1: Targeted differential display using degenerated primers.
(A): Nucleotide sequences of PCR primers encoding for the sequences present in C2 Ig domains are shown. Two primers encode for the same sequence due to the codons encoding for Ser residue. The level of degeneracy is 4096 different forms for the primers encoding YRCXAS and 2048 forms for the others. (B): The display of radioactive PCR products obtained with the YYCXAS1 primer is shown. The lanes correspond to the display of PCR product run on cDNA obtained from the t-end endothelial cell line (lane t-end), the B16 melanoma cell line (lane B16), or the coculture between the two cell lines (central lane). The arrow indicates the PCR product of interest obtained from the downregulated transcript CRAM-1 under coculture condition.

A

```
gacattcccctcgacatggcgctgagccggcggctgcgacttcgactgtacgcgcggctg      60
                 M  A  L  S  R  R  L  R  L  R  L  Y  A  R  L
cctcacttcttcctgctgctgctcttcaggggctgcatgatagaggcagtgaatctcaaa     120
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B

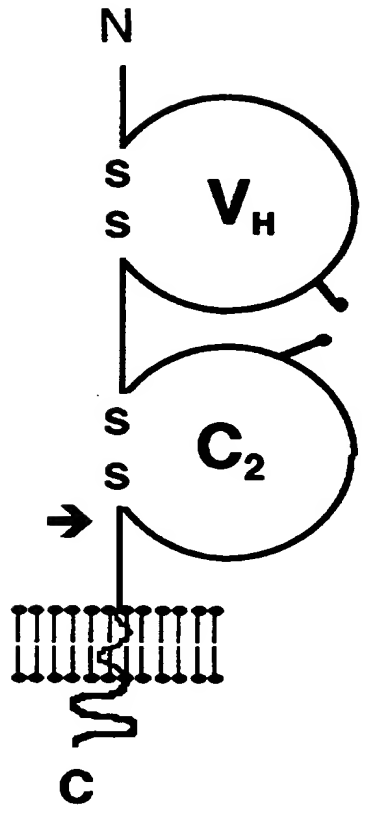


Fig 2: (A) Nucleotide and deduced amino acid sequence of Confluency Regulated Adhesion Molecule 1 (CRAM-1) cDNA. The putative hydrophobic signal peptide (first) and transmembrane region (second) are underlined. Predicted N-glycosylation sites (strikeout), Cysteines likely to form disulfide bonds (brackets) and Ser/Thr/Tyr residues of possible phosphorylation sites (bold) are indicated. (B) Structural model for murine CRAM-1 protein. Extracellular part showing a VH and a C2 like Ig domain with two putative N-linked glycosylation sites. The arrow points the region targeted by the partially degenerated primers (YYCXAS1) used in the Targeted Differential Display.

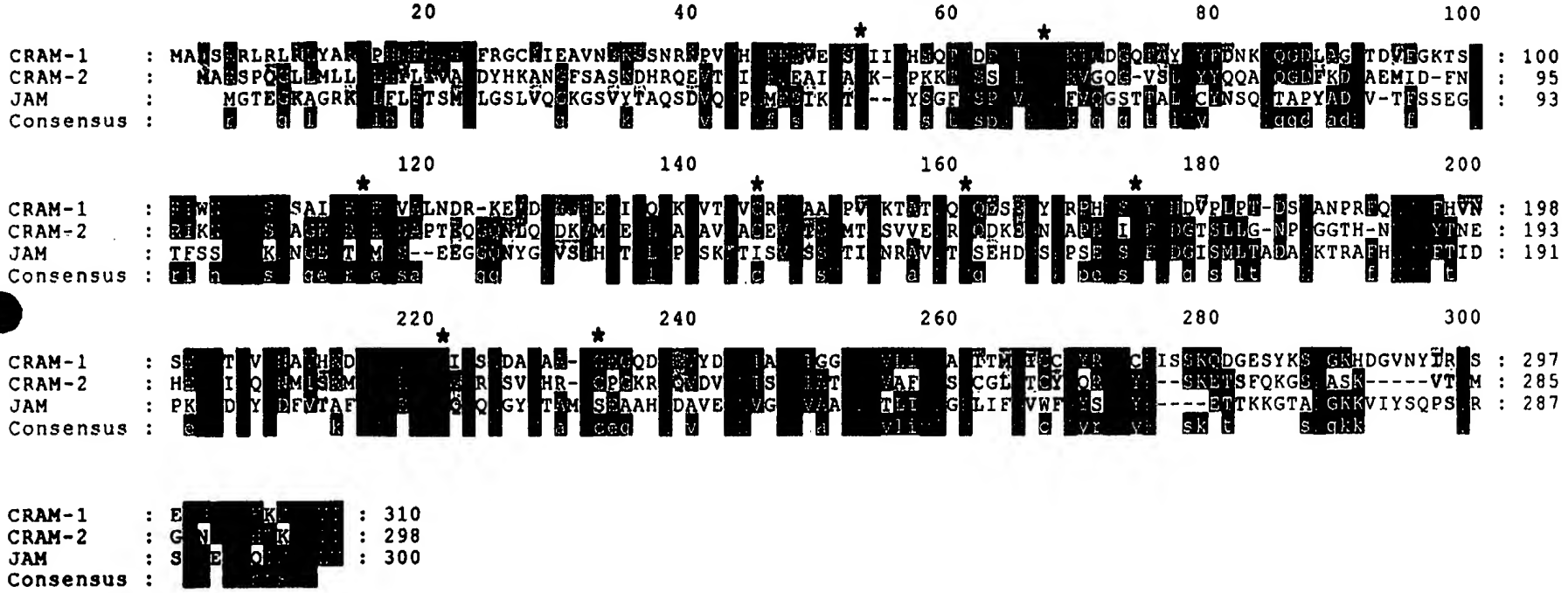


Fig 3: JAM, CRAM-1, CRAM-2 murine protein sequence alignment.
The identical residues are boxed in black and the homologous residues are shaded in gray. The overall identity is 36% between CRAM-2 and CRAM-1, is 31% between JAM and CRAM-1 and is 33% between JAM and CRAM-2; the respective homologies are 52%, 52% and 49%. The gaps are shown by dashes in the sequences. The canonical conserved residues (Cys and Trp) of the V and C2 domains are marked by an asterix.

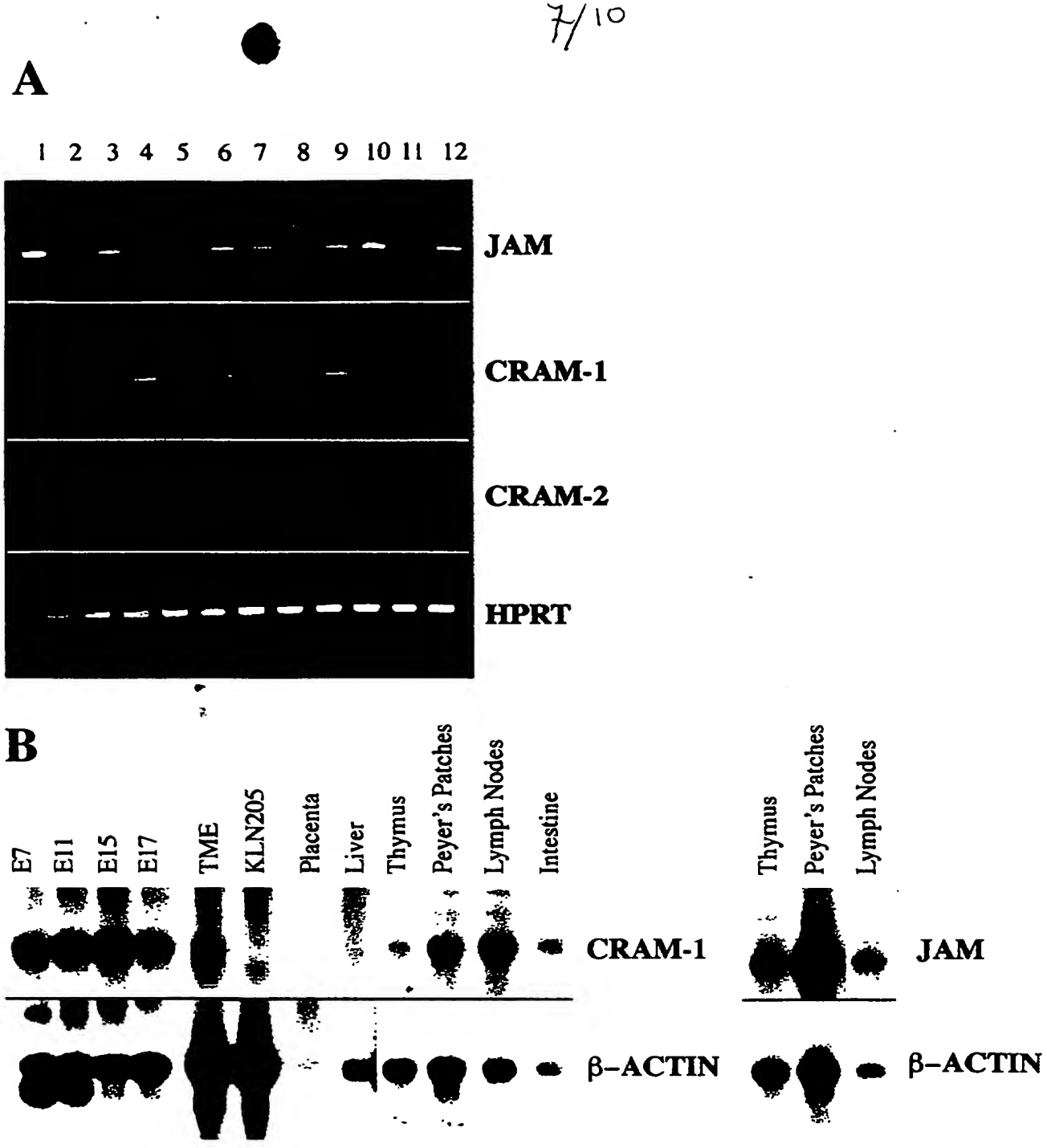


Fig 4: Expression of transcripts encoding JAM, CRAM-1 and CRAM-2 detected by RT-PCR in different cell lines (A) or detected by northern blot in various tissues (B).
(A): RT-PCR is achieved on cDNA originating from endothelial cell line treated by TNF (lanes 2 and 11 correspond to TNF treated t-end) or not treated (lanes 3, 4, 6, 7, 9, 12 correspond to b-end.5, e-end.2, t-end $V^{++}L^{-}$, t-end $V^{low}L^{++}$, TME and t-end respectively). Lanes 5 and 10 correpond to the tumor cell lines B16 (melanoma) and KLN205 (carcinoma). Lane 8 corresponds to the non transformed thymic epithelial cell line MTE4-14. Lane 1 is the positive control for JAM, CRAM-1 and CRAM-2 amplifications on the plasmids containing the cloned cDNAs. (B): Autoradiograph of P^{32} probe hybridization to mouse northern blot. The probes used for each hybridization are indicated on the left. The hybrization signals for JAM and CRAM-1 are detected at the size of 2 kb.

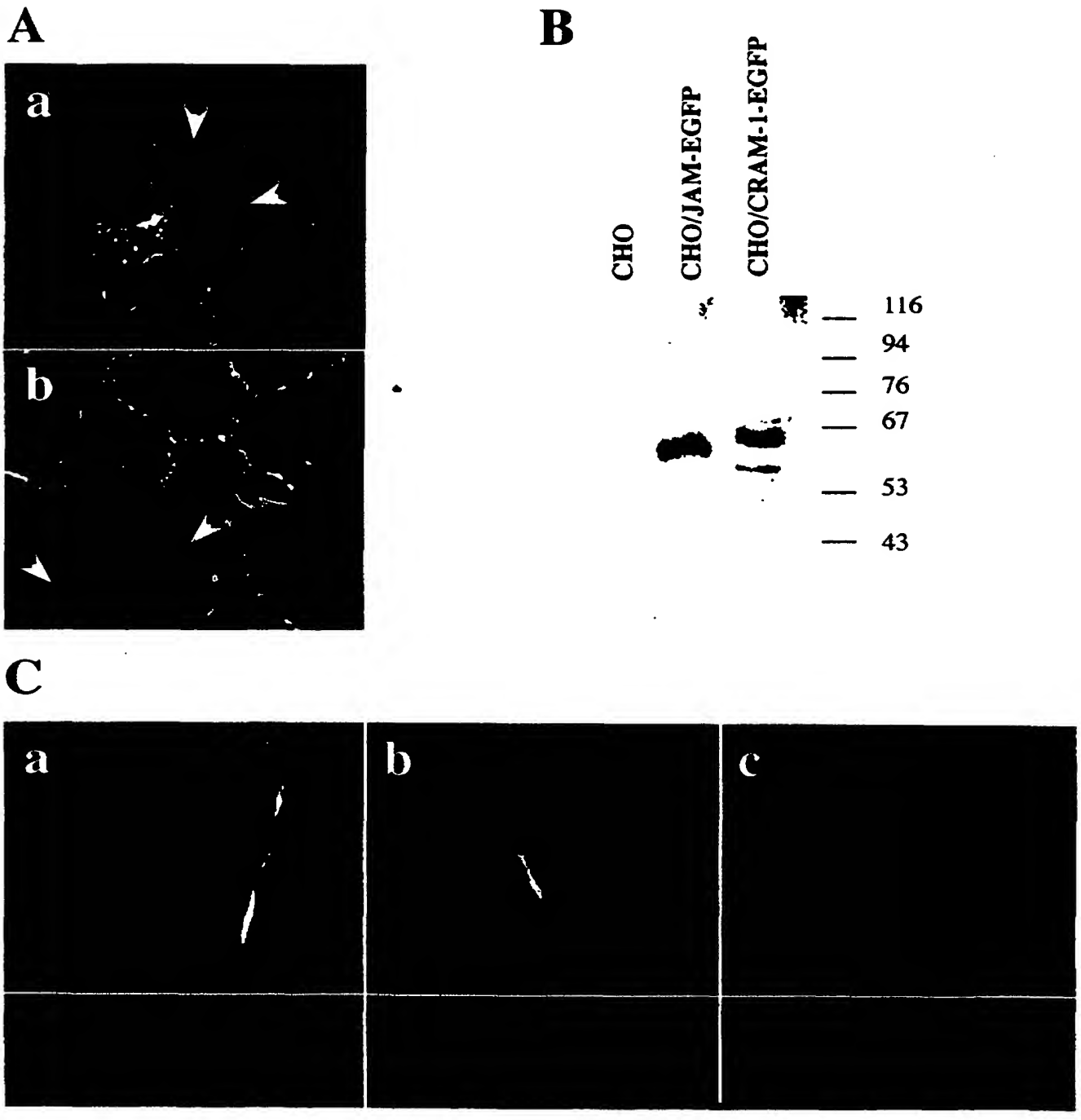
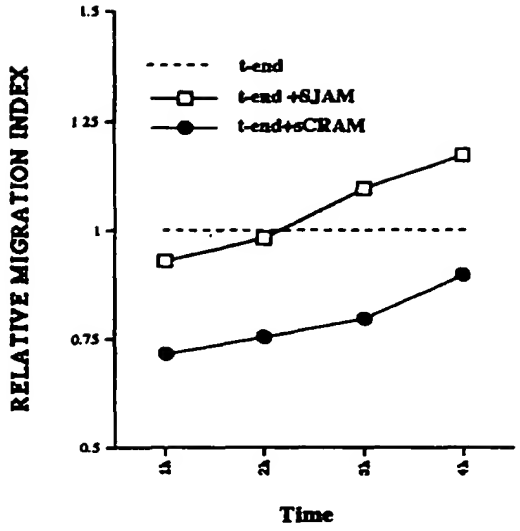


Fig 5: Immunofluorescence analysis (A and C) and immunoprecipitation (B) of EGFP chimeric molecules transfected in CHO cells.
(A) JAM-EGFP (a) and CRAM-1-EGFP (b) localization at cell-cell contacts between neighbouring transfected cells. No staining could be detected at the contact between transfected and non transfected cells (arrows). (B) Immunoprecipitation with anti EGFP pAb after surface labeling with biotine of transfected cells shown in A. The western-blot is revealed with SA-HPR and chemolumiscent substrate, MW are indicated on the right. (C) Three dimensional reconstitution of transfected CHO cell-cell contact using immunofluorescence density slicing (Improvision, Openlab system). Localization of JAM-EGFP (a), CRAM-1-EGFP (b) and CRAM-2-EGFP (c) are shown.

A



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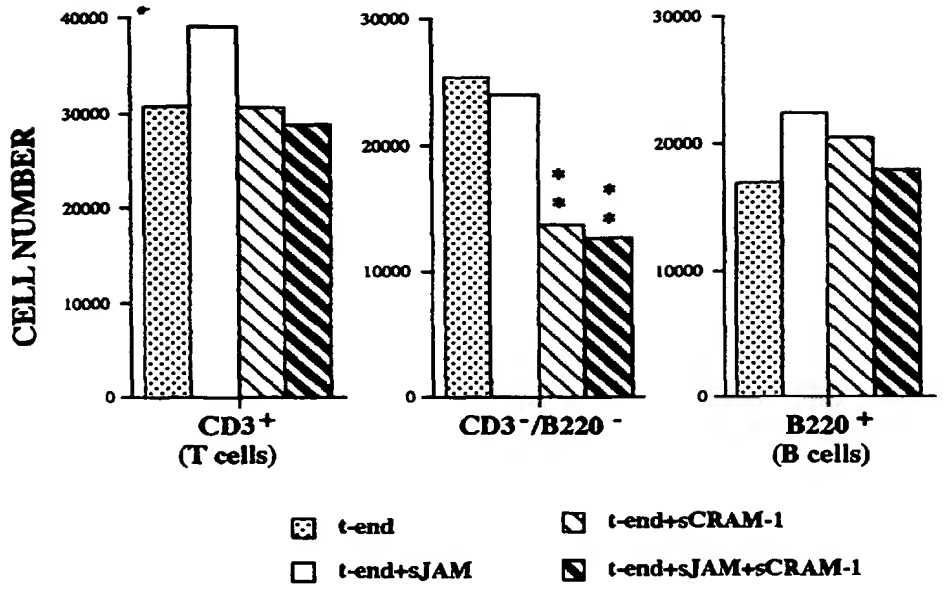

Fig 6: Effect of soluble recombinant molecules on leukocyte transendothelial migration. (A): Transmigration is expressed as a relative index and normalized on the values obtained on the non treated t-end cell line(dashed line Index 1). Results obtained in the presence of 1µg sJAM-Ig2do (open squares) or in the presence of 1µg sCRAM-1-Ig2do (filled circles) are shown. Index are calculated as a mean of five independant transmigration experiments. (B): Phenotype of transmigrated cells is expressed as cell numbers calculated from the percentages obtained by Facs analysis following staining with anti CD3-FITC and anti B220-PE. The stars indicate the experimental points with a significant difference to the controls.

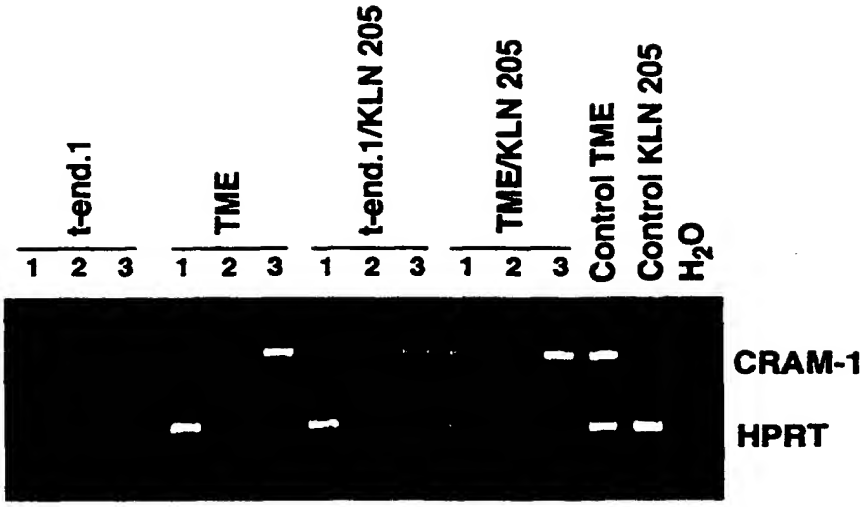

Fig 7: CRAM-1 regulation in function of confluency.
The semi-quantitative PCR is drived using a mix of primers specific for the HPRT and the CRAM-1 cDNAs. The PCR reactions are run on a 1.2% agarose gel and stained with ethydium bromide. Lanes 1, 2, 3, correspond to 100, 50 and 10% confluency respectively. A weaker signal for CRAM-1 in the 100% confluency (lanes 1) is observed. The culture condition of the endothelial cell lines (t-end.1 and TME) on their own or mixed with the tumor cell line KLN 205 is indicated.